ELAV, a *Drosophila* neuron-specific protein, mediates the generation of an alternatively spliced neural protein isoform

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Background: Tissue-specific alternative pre-mRNA splicing is a widely used mechanism for gene regulation and the generation of different protein isoforms, but relatively little is known about the factors and mechanisms that mediate this process. Tissue-specific RNA-binding proteins could mediate alternative pre-mRNA splicing. In *Drosophila melanogaster*, the RNA-binding protein encoded by the *elav* (*embryonic lethal abnormal visual system*) gene is a candidate for such a role. The ELAV protein is expressed exclusively in neurons, and is important for the formation and maintenance of the nervous system.

Results: In this study, photoreceptor neurons genetically depleted of ELAV, and *elav*-null central nervous system neurons, were analyzed immunocytochemically for the expression of neural proteins. In both situations, the lack of ELAV corresponded with a decrease in the immunohistochemical signal of the neural-specific isoform of Neuroglian, which is generated by alternative splicing. Furthermore, when ELAV was expressed ectopically in cells that normally express only the non-neural isoform of Neuroglian, we observed the generation of the neural isoform of Neuroglian.

Conclusions: *Drosophila* ELAV promotes the generation of the neuron-specific isoform of Neuroglian by the regulation of pre-mRNA splicing. The findings reported in this paper demonstrate that ELAV is necessary, and the ectopic expression of ELAV in imaginal disc cells is sufficient, to mediate neuron-specific alternative splicing.

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Received: 5 July 1996
Revised: 23 September 1996
Accepted: 8 October 1996

Current Biology 1996, Vol 6 No 12:1634–1641

Background

In *Drosophila melanogaster*, the vital *elav* (*embryonic lethal abnormal visual system*) gene is expressed pan-neurally in all stages of development. The ELAV protein is present exclusively in all immature and mature neurons [1]. Loss-of-function alleles of *elav* are embryonic-lethal, and the mutant embryos have an abnormally formed neuropil [2,3]. Hypomorphic mutations of *elav* produce aberrant eye structures, defective electroretinograms and flight defects [4,5]. The analysis of mutant clones in genetic mosaic flies has also shown a post-embryonic role for ELAV in the maintenance of photoreceptor cells, optic lobe and associated neuropil areas [5]. Thus, mutant phenotypes indicate a role for *elav* in the formation and maintenance of the nervous system.

The ELAV protein belongs to a large superfamily of RNA-binding proteins that all have one or more repeats of a well characterized RNA-binding domain (RBD; also known as the RNA-recognition motif, RRM) of approximately 80 amino acids [6,7]. Members of this superfamily are known to function in various aspects of RNA metabolism. Examples include the *Drosophila* splicing factors Sxl and Tra2, which are involved in sex-specific splicing, polyA-binding proteins, and proteins associated with snRNPs (small nuclear ribonucleoproteins) and hnRNPs (heterogenous nuclear ribonucleoproteins) [7,8]. ELAV has an alanine/glutamine-rich amino-terminal domain and three RBDs; the first two RBDs are tandem and the third RBD is separated from the first two by a hinge region [9]. ELAV-like proteins are conserved through evolution — several genes encoding proteins with three RBDs, and high homology to ELAV in these regions, have been identified in *Xenopus*, mouse, zebra fish, human and rat [10–12]. We and others have proposed that these proteins modulate expression of subsets of genes by one or more post-transcriptional mechanisms [1,12], but the absence of identified downstream targets has prevented a full understanding of their function.

The *Drosophila neuroglian* (*nrg*) gene encodes two isoforms of the Neuroglian (Nrg) protein, which are generated by alternative splicing; one of the isoforms, of 167 kDa, is expressed ubiquitously (Nrg^{167}), and the other, of 180 kDa, is expressed only in the nervous system (Nrg^{180}) [13]. In this paper, we show that *elav* regulates neural expression of the *nrg* gene by promoting the formation of the neural-specific isoform encoded by this gene. Furthermore, by expressing ELAV ectopically, we show that neural Nrg^{180} can be generated in cells that normally

express only the non-neural Nrg^{167} protein. In neurons, therefore, ELAV is necessary, and its presence in imaginal disc cells is sufficient, for the formation of the neural-specific isoform of Nrg.

Results

A novel *elav* mutation reveals that expression of neural-specific Nrg is ELAV-dependent

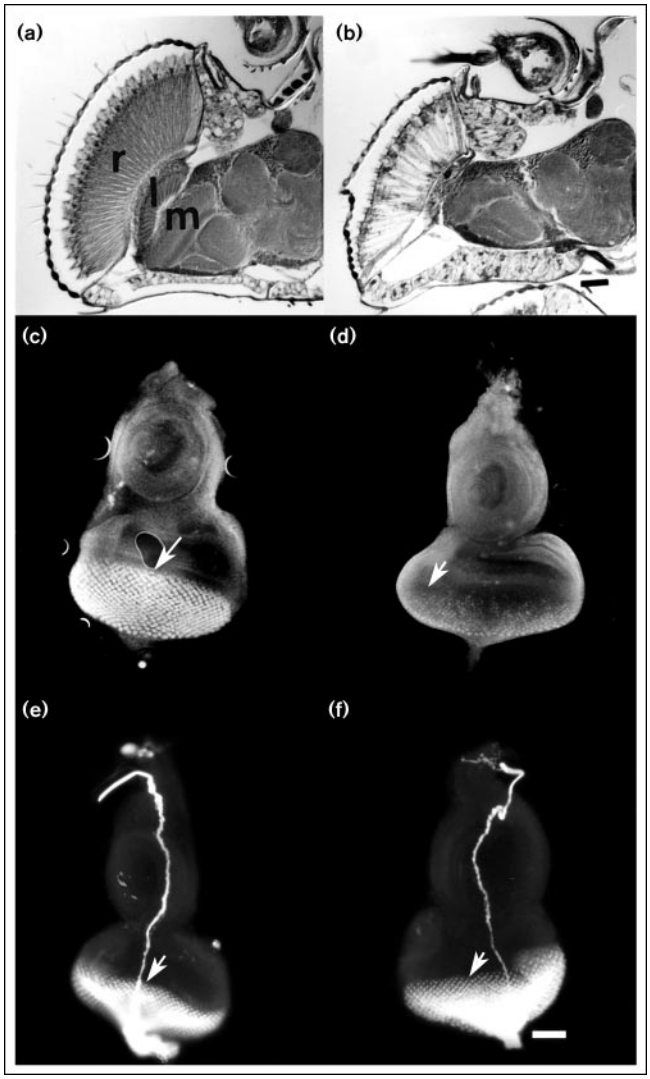
In order to identify the targets of *elav*, we analyzed the expression of candidate genes in ELAV-deficient neurons. We used a new *elav* transgene insert that encodes a wild-type ELAV protein but has a novel phenotype due to its expression pattern: *Tf(2)elav^{edr}* (*elav $^{eye\ disc\ reduced}$*) has reduced ELAV expression in photoreceptors (Fig. 1c,d), but normal expression in the central nervous system (CNS; data not shown). Thus, flies of genotype *elav^{e5}*; *Tf(2)elav^{edr}* (*elav^{e5}* is a null allele [14]) exhibited rough eyes and disorganized, vacuolar retina (Fig. 1a,b), reminiscent of the mutant eyes observed in *elav* mosaic flies [5].

We screened the expression of proteins in the ELAV-deficient eye discs using immunocytochemistry. Proteins screened included APPL [15], Chaoptin [16], Erect Wing (EWG) [17], Fas II [18], Neurotactin [19], IrreC-rst [20], Eyes absent [21], Glass [22] and neuron-specific Nrg^{180} [13]. We also used an anti-horseradish peroxidase antibody [23], which recognizes several glycosylated membrane proteins, and the monoclonal antibody 22C10 [24], which recognizes photoreceptor membranes. Among these proteins, the immunocytochemical signals for EWG (data not shown) and neuron-specific Nrg^{180} appeared to be significantly reduced in the mutant eye discs when compared with wild-type eye discs (Fig. 2b,c). In the case of other proteins, the immunocytochemical signal appeared unaffected (data not shown), implying that protein synthesis *per se* is normal in these ELAV-deficient neurons at this stage. Furthermore, the ELAV-deficient eye disc did not exhibit increased cell death, as assayed by acridine orange staining [21] (data not shown), and the gross organization of the photoreceptor clusters seemed regular (Fig. 1e,f). These observations imply that the absence of ELAV has a specific effect on the expression of EWG and Nrg^{180}, rather than a more general effect on protein synthesis.

ELAV does not affect *nrg* transcription

As the two isoforms of Nrg are generated by differential splicing of the 3′ exon [13], we investigated the expression of *nrg*. A comparison of the cDNA sequences encoding Nrg^{167} and Nrg^{180} indicated that the two isoforms are identical in the extracellular domain but differ in part of their cytoplasmic domain [13]. We cloned the genomic region corresponding to the alternatively spliced intron and mapped the alternative exons, as described in Materials and methods. As reported previously [13], a choice of 3′ splice sites allows splicing either to the upstream Nrg^{167}-specific exon or to the downstream Nrg^{180}-specific exon (Fig. 2a).

Figure 1



Phenotypic characterization of flies carrying the *elav^{edr}* transgene. (a,b) Horizontal histological sections through (a) wild-type and (b) *elav^{e5}*; *Tf(2)elav^{edr}* (ELAV-deficient) fly heads at the esophageal level. *Tf(2)elav^{edr}* is a transgene insert that provides *elav* vital function, but results in a mutant eye phenotype. Note the abnormal retina (r) and the fusion of the lamina (l) and medulla (m) in the ELAV-deficient head. Scale bar in (b) represents 50 μm in (a,b). (c,d) ELAV immunoreactivity in (c) wild-type and (d) ELAV-deficient eye disc; note the reduced staining in the ELAV-deficient eye disc. Eye discs were immunoreacted with the monoclonal antibody 9F [35], directed against ELAV. (e,f) Monoclonal antibody 22C10 immunoreactivity in (e) wild-type and (f) ELAV-deficient eye discs. 22C10 stains all photoreceptors. Note that there is no apparent difference in the signal strength and pattern in the two discs. There were no differences observed between mutant and wild-type when examined by high magnification serial sectioning using confocal microscopy (data not shown). Anterior is to the top and arrows point to the morphogenetic furrow. Scale bar in (f) represents 50 μm in (c–f).

Nrg^{180} is expressed only in neurons; Nrg^{167} is more abundant and more widely expressed, but whether it is expressed in neurons is not known [13,25]. In order to

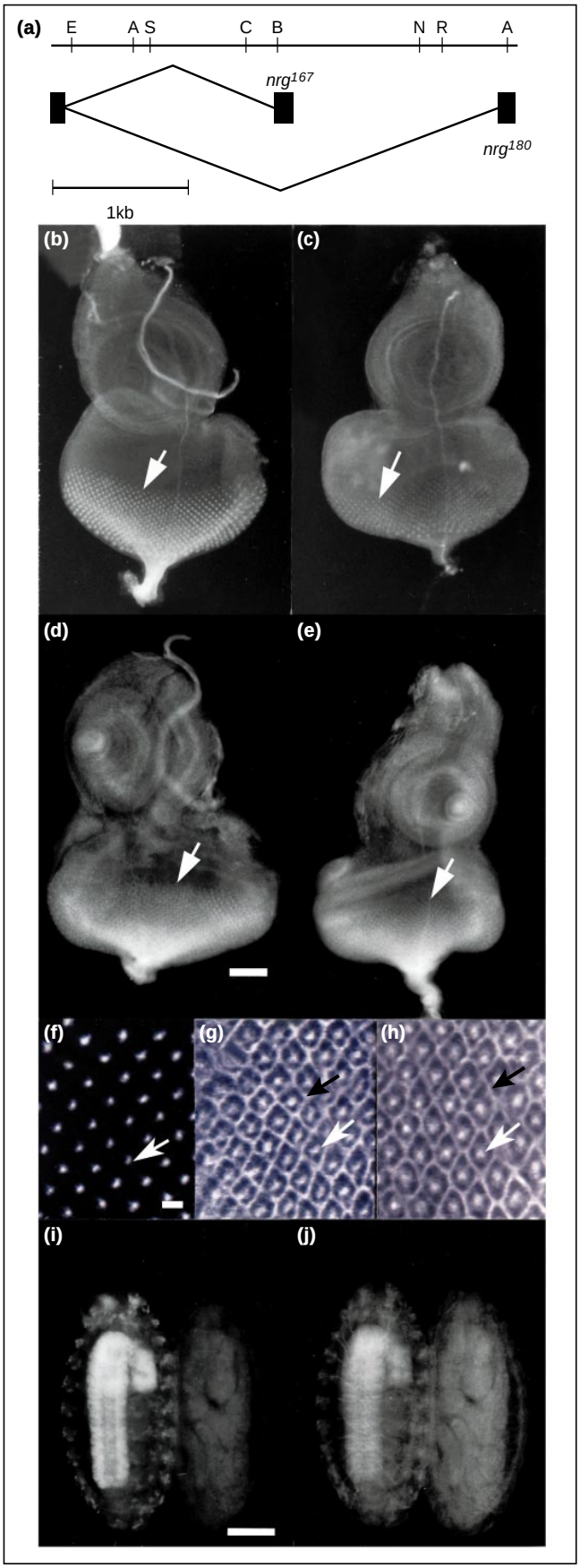


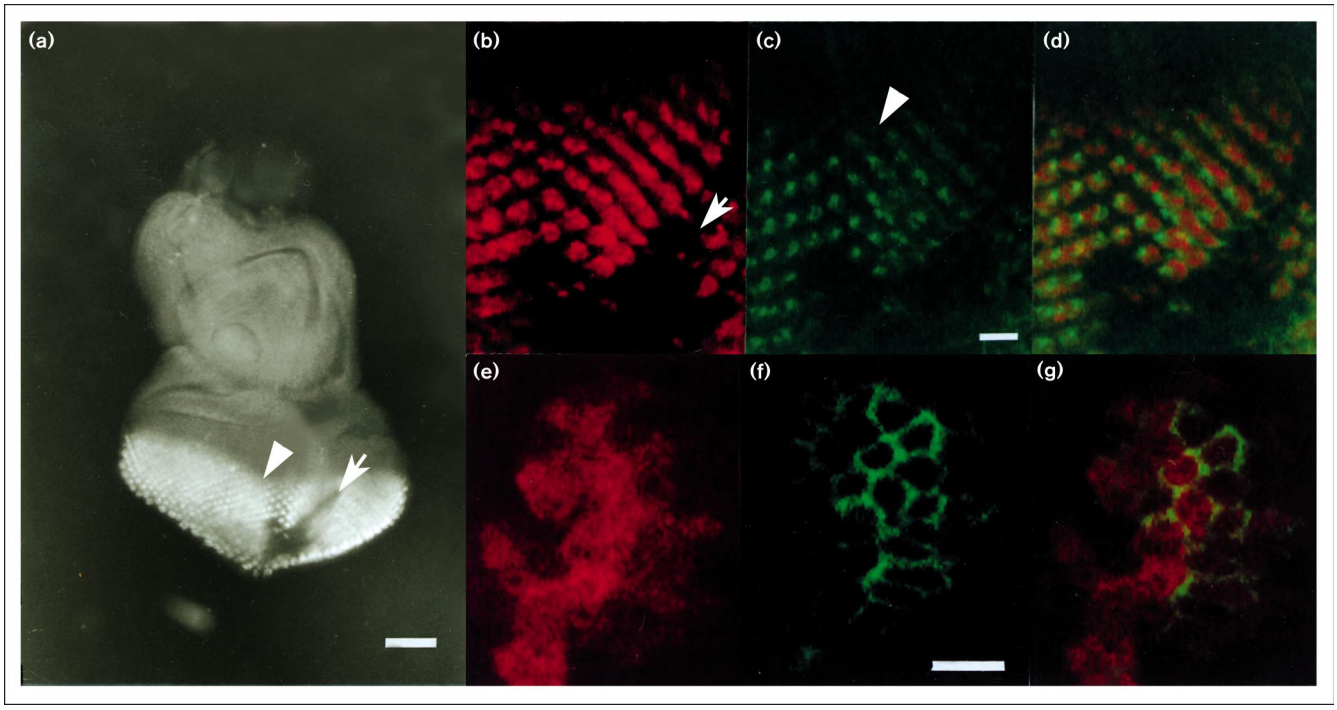
Figure 2

(a) Genomic organization of the *nrg* alternatively spliced intron. The top bar represents the genomic restriction map. Dark boxes represent exons. A choice of 3′ splice site (ss) leads to a splice to either the upstream Nrg^{167} or the downstream Nrg^{180} specific exon. Intron sizes are estimated to be 1.5 kb for the 5′ ss to the Nrg^{167} exon 3′ ss and 3.2 kb for the 5′ ss to the Nrg^{180} exon's 3′ ss. Abbreviations: E, *Eco*RV; A, *Alw*NI; S, *Sal*I; C, *Cla*I; B, *Bam*HI; N, *Nde*I; R, *Eco*RI. (b–j) Characterization of Neuroglian immunoreactivity in wild-type and ELAV-deficient mutants. In all panels, anterior is to the top; arrows in (b–e) mark the morphogenetic furrow. (b,c) Wild-type (b) and ELAV-deficient (c) eye discs immunoreacted to visualize Nrg^{180} with the monoclonal antibody BP-104; the neural-specific isoform is highly reduced in the ELAV-deficient disc. (d,e) Wild-type (d) and ELAV-deficient (e) eye discs immunoreacted with 1B7, which recognizes both Nrg^{167} and Nrg^{180}, appear to yield similar signals, implying that total Nrg levels are unaffected. The staining is all over the disc, which appears faint due to the short film exposure time chosen to reveal the differentiating ommatidia behind the morphogenetic furrow. (f–h) High-magnification confocal images of a field of ommatidia. (f) Wild-type disc immunoreacted with BP-104 shows Nrg^{180} confined to the photoreceptors, whereas both wild-type (g) and ELAV-deficient (h) discs immunoreacted with 1B7 show staining in both the photoreceptors and the epithelial cells. White arrows point to photoreceptor staining and black arrows point to the surrounding epithelial cells. The relative staining of total Nrg in neural cells relative to non-neural cells appears unchanged. These confocal sections were taken with discs immunoprocessed at the same time and under identical laser/gain settings of a Bio-Rad 600 confocal microscope. (i,j) Representative photograph showing (i) ELAV-immunoreactivity and (j) Nrg^{180}-immunoreactivity in 12–15 h embryos from a cross in which 25 % embryos are expected to be ELAV-null. Note that an ELAV-null embryo (to the right in (i)) is also Nrg^{180}-null (j). The other embryo has strong CNS (central nervous system) and PNS (peripheral nervous system) staining (+++) for ELAV and Nrg^{180}. Scale bar in (d) and (i) represents 50 μm for (b–e) and (i,j), respectively; bar in (f) represents 5 μm for (f–h).

investigate this question, we used two monoclonal antibodies: 1B7 is directed against the extracellular domain of Nrg, and can therefore detect both Nrg isoforms, whereas the epitope recognized by BP-104 is cytoplasmic, and BP-104 recognizes only Nrg^{180} in tissue extracts [13]. The specificity of BP-104 for the protein generated by the Nrg^{180} cDNA has been demonstrated in cell transfection studies: BP-104 recognizes extracts of S2 cells transfected with vectors expressing Nrg^{180} cDNA, but not those transfected with Nrg^{167} cDNA; and BP-104 does not recognize a mutated Nrg protein that lacks the cytoplasmic domain [26]. As the only difference between the two isoforms is the alternative exon [13], the epitope recognized by BP-104 is indicative of the neuron-specific splice.

As previously reported by Hortsch *et al.* [13] for wild-type eye discs, BP-104 recognized only the photoreceptors (Fig. 2f), whereas 1B7 recognized both photoreceptors and the epithelial cells of the ommatidia (Fig. 2g,h). To determine whether a reduction in Nrg^{180} in ELAV-deficient photoreceptors was due to reduced transcription of *nrg*, we tested for the expression of Nrg^{167} with 1B7. The overall signal was similar to the wild-type discs (Fig. 2d,e), and

Figure 3



Eye imaginal discs double-labeled for ELAV and Nrg^{180}. **(a)** Eye disc of the genotype *elav*^{e5}/ Y; Tf(3)*elav*^{DmORF2}/ *Ki p*^{p} (*Δ2-3, ry*$^{+}$), showing a large ELAV-null patch. Arrow in (a,b) points to the mutant patch. Scale bar represents 50 μm. **(b–d)** High-magnification confocal image through a part of *elav*-null mutant patch showing ELAV immunoreactivity in red (b) and Nrg^{180} immunoreactivity in green (c). The merged image (d) shows that ELAV-null neurons are Nrg^{180}-null. Arrowheads in (a,c) point to the morphogenetic furrow and anterior is to the top. Scale bar in (c) represents 10 μm in (b–d). **(e–g)** Ectopic expression of ELAV in wing imaginal discs double-labeled for ELAV and Nrg^{180}. High-magnification image from a wing disc of the genotype *c309/UAS-ELAV*^{2e2} showing ELAV immunoreactivity in red (e) and Nrg^{180} immunoreactivity in green (f), and a merged image (g). Note that all Nrg^{180}-positive cells are also ELAV-positive, but that not all ELAV-positive cells are Nrg^{180}-immunoreactive. In these double-labeling experiments, ELAV and Nrg^{180} were visualized using a rat polyclonal serum and BP-104, respectively. Scale bar in (f) represents 5 μm in (e–g).

the strength of the signal in the photoreceptor clusters when compared with the surrounding epithelial cells was similar in wild-type and ELAV-deficient discs (Fig. 2g,h). These observations suggest that the absence of ELAV does not affect the transcription of *nrg*, but rather that ELAV promotes formation of the neuron-specific isoform Nrg^{180}, and that in its absence only the Nrg^{167} isoform is generated.

ELAV affects the expression of Nrg^{180} in both peripheral nervous system and central nervous system neurons

To further test the dependence of Nrg^{180} on the presence of ELAV, ELAV-null clones in developing eye discs were generated by somatic mobilization [27] of a P-element carrying an *elav*$^{+}$ transgene DmORF2 [28] (see Materials and methods). Figure 3a shows a relatively large ELAV-null somatic clone, in a disc immunoreacted for ELAV (Fig. 3b) and Nrg^{180} (Fig. 3c). The merged image shows that ELAV-null clones were indeed Nrg^{180} nulls (Fig. 3d), and that ELAV acts cell-autonomously — the Nrg^{180} signal was absent only when the ELAV signal was absent.

We further tested the dependence of Nrg^{180} synthesis on ELAV in *elav*-null embryos. In the progeny of a cross expected to yield 25 % ELAV-null male embryos, single labeling with BP-104 indicated that 29 % were Nrg^{180}-null embryos; double-immunolabeling with anti-ELAV antibody and BP-104 indicated that 26 % were ELAV-null and 23 % were Nrg^{180}-null embryos (Table 1, Fig 2i,j). The small fraction of ELAV-nulls that showed weak, but above-background staining with BP-104 were likely to be the result of experimental limitations (see Table 1 legend). Double-labeling with anti-ELAV antibody and 22C10 [24] resulted in virtually all embryos being 22C10-immunoreactive, confirming the observation in eye discs (Table 1). We therefore conclude that, similar to the situation in photoreceptors, the presence of Nrg^{180} in CNS neurons correlates with the presence of ELAV.

Ectopic ELAV is sufficient to generate Nrg^{180} in tissues that normally express Nrg^{167}

If ELAV promotes formation of Nrg^{180}, perhaps the presence of ELAV in a non-neural tissue that expresses *nrg* may suffice to generate Nrg^{180}. To test this idea, the GAL4/*UAS*

Table 1

ELAV and Nrg^{180} immunoreactivity in embryos.

Progeny of cross	Antibody	Immunoreactivity	Signal strength (%) *				
			+++	++	+	$+^{1/2}$	–
1. *elav^{e5}wsn*/+ × +/*Y* *n* = 145	BP-104	Nrg^{180}	10.4	31.9	16.6	11.8	29.1
2. *elav^{e5}wsn*/+ × +/*Y*	α-ELAV and	ELAV	52.3	11.0	5.0	5.0	26.5 (15 %)†
n = 317	BP-104	Nrg^{180}	48.8	13.5	4.7	9.4	23.3
3. Wild-type	α-ELAV and	ELAV	55.8	19.8	11.2	8.2	4.8 (46 %)†
n = 269 control	BP-104	Nrg^{180}	37.1	37.1	14.1	8.5	2.9
4. *elav^{e5}wsn*/+ × +/*Y*	α-ELAV and	ELAV	27.1	41.0	5.8	0.6	25.3
n = 324 control	22C10	22C10	52.0	38.0	8.0	1.5	0.3
5. Wild-type *n* = 216 control	α-ELAV	ELAV	57.8	21.7	13.4	4.1	2.7
6. Wild-type *n* = 216 control	BP-104	Nrg^{180}	47.2	24.5	21.7	6.0	0.4

*Signal strength was assigned on a scale of +++ to – using internal comparison in a given batch of embryos. A score of +++ was given when strong CNS and PNS staining were observed, ++ for less strong staining in PNS and CNS. A score of + indicates weak CNS staining and absence of, or weak, PNS staining and a score of $+^{1/2}$ indicates very low but discernible staining in CNS after careful examination at higher magnification. †All ELAV-null embryos were independently assessed for Nrg^{180} immunoreactivity, the numbers in parenthesis refer to the percentage of ELAV-null embryos with discernible ($+^{1/2}$) Nrg^{180} immunoreactivity. 12–15 h embryos were immunoreacted, mounted and analyzed in a Zeiss inverted fluorescence microscope. 25 % of the embryos from crosses 1, 2 and 4 are expected to be genetically *elav*-null and hence have no ELAV immunoreactivity. Cross 4 demonstrates that ELAV-null embryos are 22C10-positive. The other samples – crosses 3, 5 and 6 – serve as additional controls for the uniformity of immunostaining for various antibodies and embryo populations. In cross 2, from among the ELAV-null embryos, 84 % were also Nrg^{180}-null; however, 15 % showed weak but above-background staining with BP-104. These are likely the result of experimental limitations – among the control wild-type embryos, 4.8 % were ELAV-immunonegative, and 46 % of these were Nrg^{180}-positive.

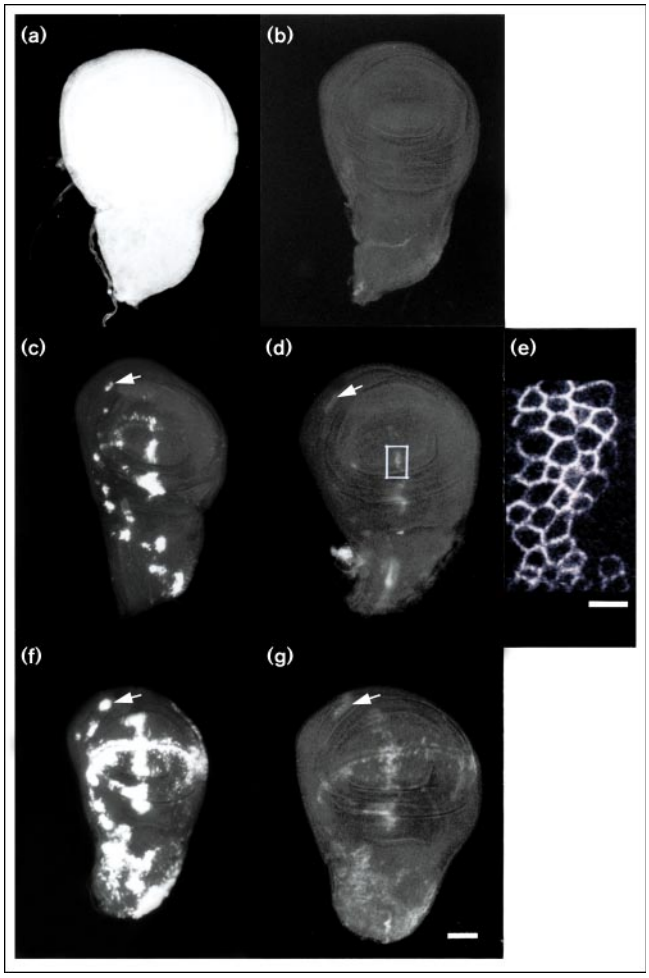
system was used to ectopically express ELAV [29]. In wild-type wing discs, Nrg^{167} was expressed ubiquitously (Fig. 4a), whereas Nrg^{180} was not expressed in early wandering third instar larvae [13] (Fig. 4b). We used a GAL4 enhancer trap line, c309, to drive the *UAS-elav* transgene in patches of wing-disc cells that do not normally express ELAV (Fig. 4c). In wing discs immunoreacted with BP-104, a signal indicative of Nrg^{180} was visible where ELAV was expressed at high levels (Fig. 4d), and higher magnifications showed that Nrg^{180} was on the cell surface (Fig. 4e). To test whether the level of Nrg^{180} produced ectopically in wing discs correlated with the concentration of ELAV, discs with two doses of *UAS-elav* transgenes were analyzed (Fig. 4f). Figure 4g shows that there was a clear enhancement in Nrg^{180} signal, which corresponded to an increase in ELAV concentration (Fig. 4f). High-magnification merged images of wing imaginal disc cells carrying *c309* and *UAS-ELAV*, and double-immunolabeled for ELAV and Nrg^{180}, showed that all Nrg^{180}-positive cells were ELAV-positive (Fig. 3e–g). As not all ELAV-positive cells were Nrg^{180}-positive, this splice must have a high threshold for ELAV in the wing imaginal disc cells.

Discussion

Taken together, the decrease or lack of Nrg^{180} when ELAV is decreased or absent, and the generation of Nrg^{180} in non-neural cells when ELAV is ectopically expressed, demonstrates that ELAV plays a key role in the generation of the neuron-specific Nrg^{180} isoform. A simple hypothesis explaining these findings is that ELAV is a trans-acting tissue-specific protein that regulates splicing of *nrg* pre-mRNA. The two *nrg* isoforms are generated by differential choice of 3′ splice sites, where the 3′ splice site used for Nrg^{167} mRNA is 5′ to the 3′ splice site used for Nrg^{180} (Fig. 2a) [13]. ELAV may therefore facilitate splicing of the Nrg^{180}-encoding mRNA by blocking the default splicing of Nrg^{167}-encoding mRNA, in a manner similar to that observed for Sxl regulation of *tra* pre-mRNA splicing in the sex-determination pathway [30]. ELAV may bind *nrg* pre-mRNA directly, or the interaction with the pre-mRNA may be indirect through other proteins involved in splicing. In vertebrates, neuron-specific splicing factors have been sought since the classic studies on alternative splicing in the calcitonin/CGRP gene [31] and the c-src gene [32], but specific cloned factors have not yet been reported.

At present we can not assess how many genes are directly modulated by *elav*, or even whether the function of ELAV is restricted to neuron-specific splicing events. It is clear, however, that *nrg* is not the only *elav*-modulated gene, as the CNS phenotype of loss-of-function alleles of *nrg* is subtle [33] compared with the loss-of-function alleles of

Figure 4



Wing imaginal discs from developing third instar larvae. (a,b) Wild-type disc stained with (a) 1B7 and (b) BP-104. 1B7 stains homogeneously throughout the disc, whereas BP-104 shows no staining; thus, only Nrg^{167} is expressed at this stage. The signal in (a) is higher than that seen in Fig. 2d,e because of increased film exposure time. (c,f) Wing discs of genotype (c) c309/*UAS-ELAV*^{2e2} and (f) c309/*UAS-ELAV*^{2e2}; *UAS-ELAV*^{3e1} immunoreacted with anti-ELAV 9F antibody. Note the increased expression of ELAV in (f) compared with (c); the arrow points to one such group of cells. (d,e) c309/*UAS-ELAV*^{2e2} and (g) c309/*UAS-ELAV*^{2e2}; *UAS-ELAV*^{3e1} wing discs stained with BP-104 to visualize Nrg^{180}. Ectopic expression of ELAV results in ectopic expression of neural-specific Nrg^{180} in non-neural cells. Arrows indicate a representative cell group that shows an increase in concentration of Nrg^{180} with an increase in ELAV concentration. (e) A high-magnification confocal image of a section – box shown in (d) – through the Nrg^{180}-expressing disc, showing the protein at the cell membrane. Scale bar in (g) represents 50 μm in (a–d, f,g); bar in (e) represents 5 μm.

elav [2,3]. It is worth noting that ELAV may influence axonal pathfinding, as *nrg*, also an essential gene, encodes a cell-adhesion molecule [25,33]. Defects in axons that form the longitudinal and transverse commisures in the embryonic neuropil are apparent in *elav*-null mutant embryos [3]. A decrease in EWG, a DNA-binding protein that is expressed pan-neurally, was also observed in ELAV-deficient eye discs; further studies are necessary to test whether ELAV affects EWG expression directly or indirectly. The phenotype of *elav* null mutants must result from misregulation of the *elav*-modulated genes that are expressed during embryogenesis.

In recent years, a number of *elav*-like genes of the RRM superfamily have been identified in *Drosophila* [14,34], human [11,35], mouse [10], *Xenopus* [10] and zebra fish [10]. Some *elav*-like proteins are found enriched in the nucleus [11,34,35], and others in the cytoplasm [36]. Many members of this subfamily show nervous system specificity [14,11,34,35]; however, ubiquitously distributed ELAV-like protein has been described in *Xenopus* and mouse [10]. *In vitro* studies with the mammalian brain-specific proteins have led to the proposal that these proteins regulate post-transcriptional processes by interacting with AU-rich 3′ untranslated regions of mRNAs [12,35]. Based on findings reported here, we suggest that tissue-specific alternative splicing is one of the functions of ELAV-like proteins.

Conclusions

The results described here show that ELAV, a *Drosophila* neuron-specific RNA-binding protein, is necessary for the generation of the neural-specific Nrg^{180} isoform in neurons. Furthermore, the ectopic expression of ELAV in non-neural cells can promote the generation of this neural-specific isoform. The identification of *nrg* as a downstream target of *elav* regulation will facilitate mechanistic understanding of ELAV function, the identification of other *elav*-regulated genes, and will help to define the process(es) regulated by *elav* in neuronal differentiation and maintenance.

Materials and methods

Fly stocks and genetic crosses

To study expression of other proteins in photoreceptors under *elav* depleted conditions, discs from larvae of the genotype *elav*^{e5}; *Tf(2)elav*^{edr} were examined. *Tf(2)elav*^{edr} (ELAV-deficient) is a transgene insertion on the second chromosome which encodes a wild-type ELAV protein, under the control of the *elav* promoter [34]. Other insertions of this transgene provide both vital and photoreceptor functions of *elav*; however, this particular insert results in an eye phenotype (our unpublished results). *elav*^{e5} is an interstitial deletion within the *elav* locus, and is *elav*-null [14].

To generate *elav*-null embryos, *elav*^{e5} *w sn*/ + virgins were crossed to wild-type *CS* males; 25 % of the progeny of this cross will be of the genotype *elav*^{e5} *w sn*/ *Y* and hence ELAV-null.

To generate *elav*-null clones in the eye disc, we used the *Tf(3)elav*^{DmORF2} transgene, which encodes a wild-type ELAV protein, in a P-element vector that provides full rescue of the *elav*^{e5} null allele [27]. *elav*-null clones in the eye discs were generated by inducing excisions of this transgene using *Ki p*^{p} (*Δ2-3 ry*$^{+}$) [36]. Female virgins of the genotype *elav*^{e5}; *Tf(3)elav*^{DmORF2}/ *TM3, Sb* were crossed to *y w*/*Y; Ki p*^{p} (*Δ2-3 ry*$^{+}$)/ *Ki p*^{p} (*Δ2-3 ry*$^{+}$) males. All male progeny of this cross are of genotype *elav*^{e5}/*Y*; *Tf(3)elav*^{DmORF2}/ *Ki p*^{p} (*Δ2-3 ry*$^{+}$) and can show Δ2-3-mediated somatic loss clones for *Tf(3)elav*^{DmORF2} transposon. Eye discs from male larvae were dissected and analyzed.

For ectopic ELAV expression, we used *UAS-ELAV^{2e2}* and *UAS-ELAV^{3e1}* transgene inserts on chromosome 2 and 3, which express *elav* cDNA under UAS control. *c309* is a GAL4 enhancer trap line with the transgene insertion on the second chromosome. Ectopic expression of ELAV was achieved by mating *UAS-ELAV^{2e2}* or *UAS-ELAV^{2e2}*; *UAS-ELAV^{3e1}* virgin females to c309 males. All progeny from this cross express ELAV ectopically.

Histology

3–5 day old male flies were fixed overnight at 4 °C in FAAG and subsequently dehydrated through an alcohol series and xylene. The heads were embedded in paraffin and 8 μm sections were cut and stained with Maeyer's hematoxylin and Pollack trichome. The detailed procedure is described in [5].

Immunohistochemistry

Imaginal discs were fixed in 4 % paraformaldehyde (pH 7.2) for 40 min. and washed several times in PBS containing 0.1 % BSA and 0.3 % Triton-X100, antibody incubations were carried out overnight at 4 °C. Immunocytochemistry for each antibody with wild-type and mutant discs was performed at the same time and, in all cases, at least 20 discs from different animals for each genotype were examined. 12–15 h staged embryos were collected, dechorionated with 50 % bleach and fixed for 30 min in 1:1 4 % paraformaldehye (pH 7.2):heptane. The embryos were devitillenized using 1:1 heptane:methanol and subsequently stored at –20 °C in methanol. Immunostaining was carried out after passing the embryos through a methanol series and finally PBT. All subsequent steps were identical to immunoproccessing of disc tissue.

Photography was carried out using a Zeiss Axiophot fluorescence microscope and Tmax-400 film. Negatives and prints were processed using identical exposures, developing conditions and magnification.

Antibodies

Monoclonal antibody 9F [37], directed against ELAV was used at 1:20, rat polyclonal directed against ELAV [1] was used at 1:200; the monoclonal antibodies BP-104 and 1B7 [13,25] were diluted 1:1, and 22C10 was used at 1:25 dilution. Secondary antibodies were bought from Jackson Immunoresearch Laboratories and used at a dilution of 1:50 or 1:100. In double-labeling experiments using embryos, ELAV was visualized using an affinity-purified rat polyclonal serum and α-rat-LRSC (minimum cross-reactive to mouse immunoglobulins); BP-104 or 22C10 were detected using a coupled system, with a secondary antibody conjugated to biotin (minimum cross-reactive to rat immunoglobulins) and streptavidin-FITC, to improve the strength of the signal. In double-labeling experiments with imaginal discs, Nrg^{180} was visualized using BP-104 and a secondary antibody conjugated to FITC, minimum cross reactive to rat immunoglobulins. All secondary antibodies were tested for the absence of cross-reactivity.

Confocal microscopy

Optical sections were taken using the Bio-Rad MRC-600 confocal microscope equipped with a krypton/argon laser. When separate samples were to be compared, they were viewed at the same time using identical laser/gain settings and printing conditions. Double-labeled imaginal discs were checked for bleed-through of signal and under the conditions used was observed to be absent. Images were processed using COMOS program and were printed on a Sony color printer.

Cloning of genomic sequences

To clone the genomic region spanning the alternatively spliced exons from cosmids containing the *nrg* locus [38], oligonucleotides named OF1, SR2 and LR2 were synthesized using published sequences [13] to serve as probes for the common exon, the Nrg^{167}-specific exon and Nrg^{180}-specific exon, respectively.

OF1 sequence: 5′ CGCTGGTCGCCAATCCGTGAGTTCAGCGAAC 3′
SR2 sequence: 5′ GCGCTGCTTACTAATTAAATCAAAGTCCTTTGCGTC 3′
LR2 sequence: 5′ TGATGCGCCGCAGCGGAATTGTTCAGTGGCTG 3′

The end-labeled oligonucleotides were used to screen, in Southern blots, a series of cosmids spanning the 7D to 7F region (Genome Mapping project, Institute of Molecular Biology and Biotechnology, Heraklion, Crete, Greece [38]). The probes hybridized to three overlapping cosmids, 168F4, 48C2, 1B2. The region spanning the intron was PCR amplified with Pwo polymerase (Boehreinger Mannheim) and cloned into pBluescipt (Stratagene) after ligating to *Xba* linkers. The cloned intron fragment was restriction mapped and the identity of the genomic region was further confirmed by partial sequencing of the exons and comparing with the published sequence [13] in this region.

Acknowledgements

We appreciate generous gift of antibodies from S. Benzer, N. Bonini, K. Fischbach, C. Goodman, I. Hariharan and G. Rubin. We thank S. Selleck and L. Manseau for the Gal4 enhancer line, A. Maloratsky for initial characterization of line c309, L. Torroja for discussions, E. Dougherty for photography, and J. Hall and M. Rosbash for critically reading the manuscript. We are grateful to A. Bieber for helpful suggestions. Some antibodies were obtained from Developmental studies hybridoma bank maintained by the Johns Hopkins University School of medicine and University of Iowa. We thank Drosophila Stock Center, Bloomington, Indiana for the Δ2-3 stock and the Genome Mapping project at the Institute of Molecular Biology and Biotechnology, Herklion, Crete, for cosmids. This work was supported by National Institute of Health grant GM 22350 and confocal microscopy was made feasible by NIH shared instrumentation grant RR 05615.

References

1. Robinow S, White K: Characterization and spatial distribution of the ELAV protein during ***Drosophila melanogaster*** development. *J Neurobiol* 1991, 22:443–461.
2. Jimenez F, Campos-Ortega JA: Genes in the subdivision 1B of the ***Drosophila melanogaster*** X-chromosome and their influence on neural development. *J Neurogenet* 1987, 4:179–200.
3. Robinow S: The ***elav*** gene of ***Drosophila melanogaster*** encodes a neuron-specific RNA binding protein which is required for the development and maintenance of the nervous system. *PhD thesis* 1989. Brandeis University:204.
4. Homyk T Jr, Isono K, Pak WL: Developmental and physiological analysis of a conditional mutation affecting photoreceptor and optic lobe development in ***Drosophila melanogaster.*** *J Neurogenetics* 1985, 2:309–324.
5. Campos AR, Grossman D, White K: Mutant alleles at the locus ***elav*** in ***Drosophila melanogaster*** lead to nervous system defects. A developmental genetic analysis. *J Neurogenetics* 1985, 2:197–218.
6. Burd CG, Dreyfuss G: Conserved structures and diversity of functions of RNA-binding proteins. *Science* 1994, 265:615–621.
7. Kenan DJ, Query CC, Keene JD: RNA recognition: towards identifying determinants of specificity. *Trends Biochem Sci* 1991, 16:214–216.
8. Mattaj IW: A binding consensus: RNA-protein interactions in splicing, snRNPs, and sex. *Cell* 1989, 57:1–3.
9. Robinow S, Campos AR, Yao KM, White K: The ***elav*** gene product of ***Drosophila***, required in neurons, has three RNP consensus motifs. *Science* 1988, 242:1570–1572.
10. Good PJ: A conserved family of ***elav***-like genes in vertebrates. *Proc Natl Acad Sci USA* 1995, 92:4557–4561.
11. Szabo A, Dalmau J, Manley G, Rosenfeld M, Wong E *et al.*: HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal. *Cell* 1991, 67:325–333.
12. King PH, Levine TD, Fremeau, RT, Keene JD: Mammalian homologs of ***Drosophila*** Elav localized to a neuronal subset can bind ***in vitro*** to the 3′ UTR of mRNA encoding the Id transcriptional repressor. *J Neurosci* 1994, 14:1943–1952.
13. Hortsch M, Bieber AJ, Patel NH, Goodman CS: Differential splicing generates a nervous system-specific form of ***Drosophila*** Neuroglian. *Neuron* 1990, 4:697–709.
14. Yao KM, Samson ML, Reeves R, White K: Gene ***elav*** of ***Drosophila melanogaster***: a prototype for neuronal-specific RNA binding protein gene family that is conserved in flies and humans. *J Neurobiol* 1993, 24:723–739.
15. Luo LQ, Martin-Morris LE, White K: Identification, secretion, and neural expression of APPL, a ***Drosophila*** protein similar to human amyloid protein precursor. *J Neurosci* 1990, 10:3849–3861.

16. Zipursky SL, Venkatesh TR, Benzer S: From monoclonal antibody to a gene for a neuron-specific glycoprotein in ***Drosophila.*** *Proc Natl Acad Sci USA* 1985, 82:1855–1859.
17. DeSimone SM, White K: The ***Drosophila erect wing*** gene, which is important for both neuronal and muscle development, encodes a protein which is similar to the sea urchin P3A2 DNA-binding protein. *Mol Cell Biol* 1993, 13:3641–3649.
18. Grenningloh G, Rehm EJ, Goodman CS: Genetic analysis of growth cone guidance in ***Drosophila***: Fasciclin II functions as a neuronal recognition molecule. *Cell* 1991, 67:45–57.
19. Hortsch M, Patel NH, Bieber AJ, Traquina ZR, Goodman CS: ***Drosophila*** neurotactin, a surface glycoprotein with homology to serine esterases, is dynamically expressed during embryogenesis. *Development* 1990, 110:1327–1340.
20. Schneider T, Reiter C, Eule E, Bader B, Lichte B *et al.*: Restricted expression of the irreC-rst protein is required for normal axonal projections of columnar visual neurons. *Neuron* 1995, 15:259–271.
21. Bonini NM, Leiserson WM, Benzer S: The ***eyes absent*** gene: genetic control of cell survival and differentiation in the developing ***Drosophila*** eye. *Cell* 1993, 72:379–395.
22. Ellis MC, O'Neill EM, Rubin GM: Expression of ***Drosophila*** glass protein and evidence for negative regulation of its activity in non-neuronal cells by another DNA-binding protein. *Development* 1993, 119:855–865.
23. Snow PM, Patel NH, Harrelson AL, Goodman CS: Neural-specific carbohydrate moiety shared by many surface glycoproteins in ***Drosophila*** and grasshopper embryos. *J Neurosci* 1987, 7:4137–4144.
24. Zipursky SL, Venkatesh TR, Teplow DB, Benzer S: Neuronal development in the ***Drosophila*** retina: monoclonal antibodies as molecular probes. *Cell* 1984, 36:15–26.
25. Bieber AJ, Snow PM, Hortsch M, Patel NH, Jacobs RJ, Traquina ZR, *et al.*: ***Drosophila*** Neuroglian: a member of the immunoglobulin superfamily with extensive homology to the vertebrate neural adhesion molecule L1. *Cell* 1989, 59:447–460.
26. Hortsch M, Wang YE, Marikar Y, Bieber AJ: The cytoplasmic domain of the ***Drosophila*** cell adhesion molecule is not essential for its homophilic adhesive properties. *J Biol Chem* 1995, 270:18809–18817.
27. Robertson HM, Preston CR, Phillis RW, Johnson-Schlitz D, Benz WK, Engles WR, *et al.*: A stable source of P-transposase in ***Drosophila melanogaster***. *Genetics* 1988, 118:461–470.
28. Yao KM, White K: Organizational analysis of ***elav*** gene and functional analysis of ELAV protein of ***Drosophila melanogaster*** and ***Drosophila virilis.*** *Mol Cell Biol* 1991, 11:2994–3000.
29. Brand AH, Perrimon N: Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 1993, 118:401–415.
30. Sosnowski BA, Belote JM, McKeown M: Sex-specific alternative splicing of RNA from the transformer gene results from sequence-dependent splice site blockage. *Cell* 1989, 58:449–459.
31. Leff SE, Evans RM, Rosenfeld MG: Splice commitment dictates neuron-specific alternative RNA processing in Calcitonin/CGRP gene expression. *Cell* 1987, 48:517–524.
32. Martinez R, Mathey-Prevot B, Bernards A, Baltimore D: Neuronal pp60c-src contains a six-amino acid insertion relative to its non-neuronal counterpart. *Science* 1987, 237:411–415.
33. Hortsch M, Goodman CS: Neuroglian. In *Guidebook to the Extracellular Matrix and Adhesion Proteins.* Oxford University Press; 1993:161–163.
34. Kim YJ, Baker BS: The ***Drosophila*** gene ***rbp9*** encodes a protein that is a member of a conserved group of putative RNA-binding proteins that are nervous system-specific in both flies and humans. *J Neurosci* 1993, 13:1045–1056.
35. Levine TD, Gao F, King PH, Andrews LG, Keene JD: Hel-N1: an autoimmune RNA-binding protein with specificity for 3′ uridylate-rich untranslated regions of growth factor mRNAs. *Mol Cell Biol* 1993, 13:3494–3504.
36. Gao F-B, Keene JD: Hel-N1/Hel-N2 proteins are bound to poly(A)$^+$ mRNA in granular RNP structures and are implicated in neuronal differentiation. *J Cell Sci* 1996, 109:579–589.
37. O'Neill EM, Rebay I, Tijan R, Rubin GM: The activities of two Ets-related transcription factors are required for ***Drosophila*** eye development are modulated by the Ras/MAPK pathway. *Cell* 1994, 78:137–147.
38. Madueno E, Papagiannakis G, Rimmington G, Saunders RDC, Savakis C, Siden-Kiamos I, *et al.*: A physical map of the X-chromosome of Drosophila melanogaster: cosmid contigs and sequence tagged sites. *Genetics* 1995, 139:1631–1647.